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BRIEF REPORTS

Deterioration of expanded polystyrene caused by *Aureobasidium pullulans* var. *melanogenum*

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Abstract An expanded-polystyrene factory located in northern Buenos Aires reported unusual dark spots causing esthetic damage in their production. A fungal strain forming black-olive colonies on extract malt agar medium was isolated from the damaged material and identified as *Aureobasidium pullullans* var. *melanogenum*. This fungus is particularly known for its capacity to produce hydrolytic enzymes and a biodegradable extracellular polysaccharide known as pullulan, which is used in the manufacture of packaging material for food and medicine. Laboratory tests were conducted to characterize its growth parameters. It was found that the organism was resistant to a wide range of pHs but did not survive at temperatures over 65 °C. The proposed action plan includes drying of the material prior to packaging and disinfection of the machinery used in the manufacturing process and of the silos used for raw material storage.

PALABRAS CLAVE
Industria;
Manejo;
Mancha

Deterioro de poliestireno expandido causado por *Aureobasidium pullulans* var. *melanogenum*

Resumen Una fábrica de poliestireno expandido situada en el norte de Buenos Aires reportó casos inusuales de manchas oscuras que causaban un daño estético en su producción. A partir del material dañado se aisló una cepa de hongo que forma colonias negro-oliváceas en medio agar-malta y que fueron identificadas como *Aureobasidium pullullans* var. *melanogenum*. Este hongo es particularmente conocido por su capacidad de producir enzimas hidrolíticas y un

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polisacárido extracelular biodegradable, el pululano, utilizado para la fabricación de envases para alimentos y medicinas. Se llevaron a cabo ensayos de laboratorio para caracterizar sus parámetros de crecimiento. Se encontró que el organismo es resistente a un amplio rango de pH, pero no sobrevive a temperaturas superiores a 65 °C. El plan de acción propuesto incluye el secado del material antes de su envasado y la desinfección tanto de la maquinaria utilizada en el proceso de fabricación como de los silos utilizados para el almacenamiento de la materia prima.

Expanded polystyrene (EPS) is commonly used in the manufacture of food packaging, the protection of fragile and delicate products and as a thermal insulator. Particulate polystyrene (beads), generally containing pentane as blowing agent, is used as feedstock for manufacturing these products. The first step of the process consists of preexpansion of these beads, which, in this case, are then stored in silos. They rest until the molding process, when they are thermally forced to expand again and bound to each other inside the mold.[1]

The use of this material has many advantages such as low density, moisture resistance and shock absorption capacity, but its most notable feature is its hygiene as there is no source of nutrition for any microorganism.[2]

Aureobasidium pullulans (de Bary) G. Arnaud is a worldwide distributed fungus, usually known as ''black yeast''.[5] It is a dimorphic species, with high phenotypic plasticity, it has the ability to respond to environmental signals by altering its morphology, physiological state and/or behavior; this characteristic allows it to survive in different environments.[10] This fungus is particularly known for its capacity to produce a large number of hydrolytic enzymes[4] and a biodegradable extra cellular polysaccharide known as pullulan (poly-α-1,6-maltotriose), used in the manufacture of packaging for food and medical products.[9] There are three described varieties of *A. pullulans*: *A. pullulans* var. *pullulans*,[11] *A. pullulans* var. *melanogenum*[5] and *A. pullulans* var. *aubasidani* Yurlova.[13] *A. pullulans* is found in a large variety of habitats, in soil, fresh and salt water and ice,[14] as a contaminant in aviation fuel,[6] spacecraft[3] and even in damaged nuclear reactors.[15] It was isolated from a variety of surfaces including glass[7] and painted surfaces.[8] According to Webb et al.[12], *A. pullulans* is involved in the early stages of biodeterioration of materials such as plasticized vinyl chloride. However, up to date no records of this fungus growing on polystyrene are available.

The aim of this work was to study the causal agent of the deterioration of spoiled EPS samples, suggesting possible practices for its elimination.

Samples were obtained from two types of materials: packaging for the food industry and raw materials. Packaging showed stains on its surface with different shades, ranging from black to brown, which appeared on the packaging shortly after its manufacture during the storage period (Fig. 1A and B). The raw materials (EPS beads) were obtained from the storage tank and were superficially sterilized with alcohol 70% and hypochlorite 2% and inoculated in Petri dishes containing MEA medium (malt extract 12.7 g l^{-1}, glucose 10 g l^{-1}, agar 20 g/l) to stimulate mycelial growth. The surface of the spotted packaging was sterilized, using alcohol 70% and hypochlorite 2%, and EPS samples were extracted at two depths beneath the surface of the material (0.5 and 1 cm). They were inoculated in Petri dishes with MEA. These Petri dishes were incubated at 28 °C for 15 days. Cultures were monitored daily.

Cultures obtained from inoculated Petri dishes were isolated and a reinoculation test was conducted in new EPS material. Moist chambers were constructed to achieve favorable conditions for fungal colonization. From this new EPS material the microorganism was recovered, identified and the strain is available under MEX2115 in the culture collection of the MEX (Experimental Mycology) laboratory (FCEN – UBA). Controls of uninoculated material were kept in the same moist chambers. In order to verify if polystyrene was used as the source of carbon and energy by our strain, dry weight losses were measured against the controls after 3 months, since previously dried materials did not show colonization.

Samples from EPS material and cultures were observed under light microscope (LM) and scanning electron microscope (SEM EDS INCA ENERGY, Oxford Instruments Scanning electron microscope with Field Emission Gun (FEG) Zeiss DSM 982 GEMINI secondary electrons detector in-lens). At least 20 measurements were made to estimate the size of microscopic elements.

Sterile water was used to recover spores from a 14-day culture grown in Petri dish with MEA. This assay does not distinguish between types of spores, since the main goal of the experiment is to study the propagules as dispersal units. The spore suspension was fractionated and exposed to a range of temperatures (50, 65, 80 and 100 °C) for 0.5; 1; 10; 30; 60 and 120 min. Samples were cooled down and inoculated on MEA. After 10 days, the cultures were checked for viability. Cultures with 7 days of growth were exposed to temperatures of 37, 50 and 80 °C in ovens for 0.25; 0.5; 1; 2 and 4 h. Colonies were replicated in a new MEA plate in order to evaluate viability. To test the growth at different pH levels, MEA media with phosphate buffer (pH 6, 7 and 8) and citrate–phosphate buffer (pH 2.6, 3, 4, 5 and 6) were inoculated with agar plugs of 5 mm diameter, provided from 7-day old cultures grown on MEA and incubated at 28 °C. To discard a possible effect of the buffer, the pH 6 culture was separately replicated using both buffers.

To test the resistance of the microorganism to common industrial disinfectants, agar plugs were taken from cultures grown in MEA and placed in test tubes containing sodium hypochlorite (NaClO) or hydrogen peroxide (H_2O_2)

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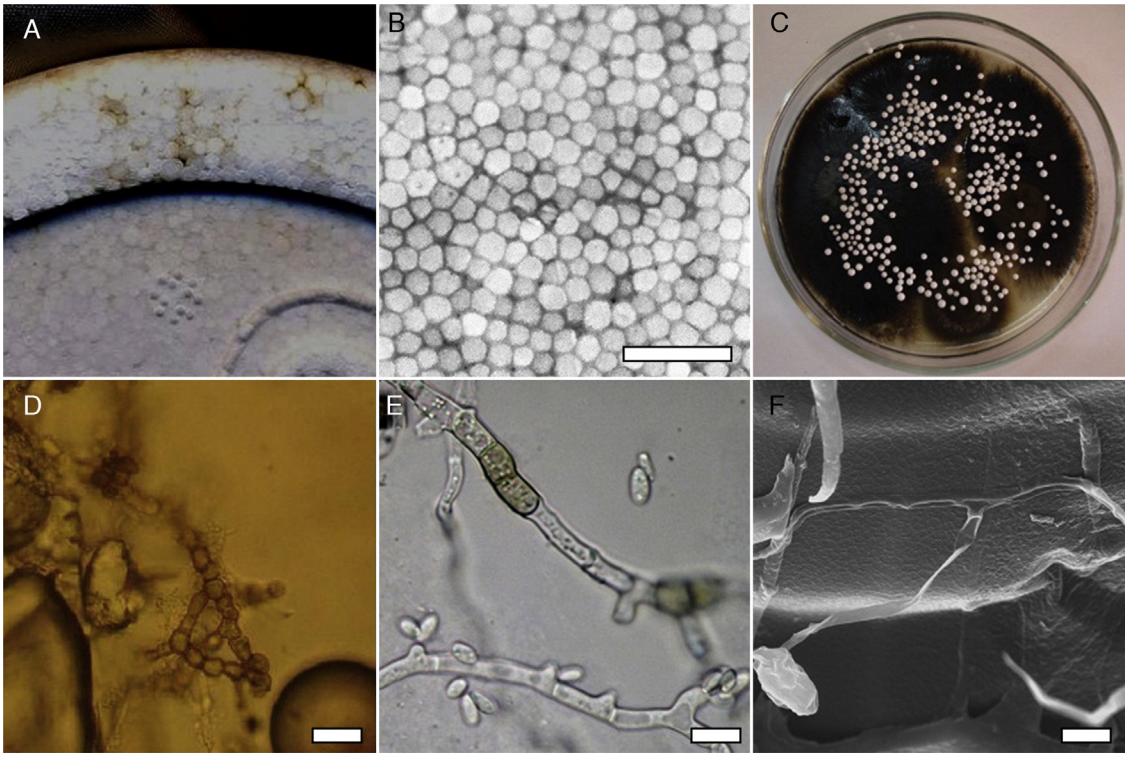

Figure 1 (A and B) Stained polystyrene products. (C) Colony emerging from unexpanded beads in MEA. (D) Mycelium observed under light microscope in the expanded material. (E) Structures observed in pure culture incubated for 30 days at 28 °C in MEA showing conidiogenous cells, conidia, and melanized cells. (F) Superficial colonization of the beads by hyphae under SEM. Scale bar (B) = 1 cm and (D–F) = 10 μm.

at different concentrations. After 5 min the agar plugs were removed and incubated for 7 days at 28 °C in MEA.

Olive-black colonies were isolated from samples of EPS beads, from superficial spots on the manufactured material and at 0.5 and 1 cm below the surface. Based on their morphological characteristics the microorganism was identified as *A. pullulans* var. *melanogenum*. Reinoculation assays confirmed that this organism is responsible for the spots found in the material. Microscopic observations showed that this fungus grows in the surface of EPS beads but not in the inside. Cultures made with EPS beads, with and without surface sterilization, corroborate this fact, since superficial sterilization completely prevented fungal growth.

The isolate of *A. pullulans* var. *melanogenum* obtained was able to grow over a wide pH range (2.5–8). Thermal death assay (Table 1) showed that the mycelium was capable of maintaining its viability up to 1 min at 50° C. Spores suspension also showed moderate resistance, maintaining the activity after thermal treatments at up to 65 °C for 30 s.

Table 1 Thermal death test. Controls were kept at room temperature. Assays were performed in triplicate.

	Temperature (°C)						
	Spores[a]				Agarized cultures[b]		
Time	50	65	80	100	37	50	80
0	+++	+++	+++	+++	+++	+++	+++
30″	++	+	+	–	+++	++	–
1′	+	–	–	–	+++	+	–
10′	–	–	–	–	+++	–	–
30′	–	–	–	–	+++	–	–
1 h	–	–	–	–	+++	–	–
2 h	–	–	–	–	+++	–	–

[a] Colony density; (+): infrequent colonies, (++): frequent but isolated colonies, (+++): colonies covering the entire surface of the plate, (–) null growth.
[b] Mycelial density; (+), (++), (+++) = increasing mycelial density; (–) null growth.

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Table 2 Resistance to treatment with disinfectants. Assays were performed in triplicate.

Hydrogen peroxide		Sodium hypochlorite	
Concentration (%)	MD	Concentration (%)	MD
0	+++	0	+++
0.2	+++	0.1	++
0.5	+++	0.25	–
1	+++	0.5	–
2	++	0.75	–
5	–	1	–
10	–		
20	–		

MD = mycelial density; (+), (++), (+++) = increasing mycelial density; (–) null growth.

The result of the test, which was carried out with disinfectant substances, showed that the fungus died after 5 min in sodium hypochlorite 0.1 %, resisting concentrations of hydrogen peroxide higher than 2 % (Table 2). Mycelium density is used to subjectively estimate the surviving inoculum, since the yeast/mycelium morphology of this organism does not allow proper colony counting.

The analysis of the cultures obtained from the reinoculation in new EPS material, with strains isolated from the stained material long-established that *A. pullulans* var. *melanogenum* is the causative organism of the spotting. The growth of colonies on humid material stopped after a few days, and no significant dry weight losses were detected even after 3 months of culture in humid chambers. However, the precise composition of the feedstock is not specified by the supplier; it usually contains various chemical additives, all of which are potentially biodegradable, that may be used by the fungus as a nutrient source.

Cultural characteristics: Colonies of *A. pullulans* var. *melanogenum* on MEA growing at a temperature of 28 °C, reached a diameter of 30 mm in 7 days, have got a black olive coloration with arachnoids margins and a smooth, slimy surface (Fig. 1C).

Microscopy: Hyphae hyaline, thin-walled, septate. Conidiogenous cell undifferentiated, intercalary or terminal on hyaline hyphae. Conidia production occurred almost synchronously forming dense groups. Conidia hyaline, one-celled, smooth, ellipsoidal, very variable in shape and size (7–16 μm × 4–6 μm). Brown vegetative hyphae septate, later dismantled in chlamydospores, were observed. Chlamydospores thick-walled, dark brown, one (13–16 μm × 7–11 μm) or two celled (17–23 μm × 7–12 μm) (Fig. 1D and E).

A. pullulans var. *melanogenum* produces many propagules that are easily dispersed by air. This fact, combined with its ability to grow in different environments, may be the cause of its global distribution: it has been found on every continent and was isolated from many different environments. This feature also contributes to the difficulty in controlling a contamination with this fungus. The characterization of this new isolate provides some guidelines for the management and control of this fungus. The pH assay showed that this strain was viable in a range of 2.5–8; therefore, this is not an option to control contaminations since the water used in the manufacturing processes is maintained in the range of pH 6–7. Regarding the thermal resistance, this strain proved to be more resilient than the one reported by Zalar et al.[14] who stated that the maximum temperature tolerated by *A. pullulans var. melanogenum* was 35 °C. Based on the results obtained, including a drying step in oven for at least 1 min at 65 °C seems to be a feasible option to remove fungal propagules. However, the addition of a step to the production line often translates into an increase in costs and production time; therefore, it is necessary to calculate the cost–benefit ratio.

The use of disinfectants seems a more feasible option to reduce the population of propagules in storage tanks, machinery and water pipes. Sodium hypochlorite is easily manageable, inexpensive and degrades in contact with air without residues. *A. pulullans* var. *melanogenum*, like most fungi, is highly sensitive to this substance and a concentration of 0.25 % is sufficient to eliminate this organism.

Since counting of colony forming units was high in the stored material, the complete emptying of tanks would be the critical point of the cycle of infection.

Methods of exposure to ultraviolet light are not recommended since strongly melanized spores of *A. pulullans* var. *melanogenum* are resistant to long radiation exposures.

In conclusion, based on the characteristics of this fungus and the results of this study, we conclude that the most appropriate approach in this case is to use sodium hypochlorite at a concentration of 0.25 % as *in situ* management practice to control and prevent damage of EPS material. Furthermore, since this fungus grows in conditions of high humidity, the drying of the finished product into a furnace of at least 50 °C is recommended.

SEM images of infected expanded material revealed that the colonization was restricted to the bead surface. No active hyphal penetration was observed (Fig. 1F).

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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